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(54) Title: METHOD OF CULTURING CELLS

(57) Abstract

Cells are cultured in a continuous culture system wherein glucose delivery and pH maintenance are based on values determined in a static culture of the cell line of interest. To provide maximum growth of the cell line in the continuous culture system, a pH level is maintained in the continuous culturing system corresponding to a pH value occurring at a point in time of greatest percentage increase in cell number in a static culture of the cell line of interest. The glucose and lactate levels are maintained in the continuous culture system corresponding to glucose and lactate levels at a point in time of greatest quantitative increase in cell number in the static culture of the cell line of interest. If the cell line of interest is a cell line used to produce a useful biological product, then the pH set point is changed at a maximum cell count to a value corresponding to a pH value occurring at a time wherein the cell count is at a maximum in the static culture and the glucose and lactate set points are changed to values corresponding to a point in time where the production of the biological product per cell per time period is greatest in the static culture.

> 15 g/L glucose (Fig. 1)

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METHOD OF CULTURING CELLS

BACKGROUND OF THE INVENTION

1. Field of the Invention.

The present invention relates to cell culturing, and in particular, it relates to the control of glucose delivery and pH and lactate maintenance in a continuous culturing system.

2. Description of the Prior Art.

Continuous culturing systems have been found to be highly successful in culturing cells for achieving both high cell densities and for providing environmental conditions to cells, such as mammalian cells. Mammalian cells, in turn, have recently been employed to produce useful biological products, such as insulin, interferons and other proteins or particles.

One highly successful continuous culturing system is the ACUSYST-P cell culturing system manufactured by Endotronics, Inc. of Coon Rapids, Minnesota. This system employs the use of hollow fiber cartridges wherein a medium containing nutrients and other factors is delivered through the lumens of the hollow fibers and cells are cultured in an extracapillary space (ECS) between the hollow fibers and the inside surface of the cartridge walls. Nutrients and other factors pass through the semipermeable membrane wall of the hollow fibers into the ECS while waste products pass from the ECS through the semipermeable membrane walls into the media and are carried away. If the cell line produces a useful biological product, this is

retained in the cell culturing space due to the selected porosity of the hollow fiber membrane.

Presently, such continuous cell culturing systems provide glucose and maintain pH and lactate at set points that have been emperically determined for a cell line of interest and then kept constant throughout the growth phase of the cell, the production phase of the cell line and the death phase of the cell line.

Static culturing systems, which were in use well prior to the hollow fiber continuous culturing systems, provide glucose and maintain pH and lactate using a cruder approach that involves removing media from the static culturing vessel at a selected time interval, such as once a day, and then replacing that media.

With the advent of commercialization of cell culturing, a need has arisen for developing an environment for more efficient growth of cells and for higher rates of production of biological products from those cells.

SUMMARY OF THE INVENTION

The present invention includes a method of culturing cells wherein set point values for pH, glucose and lactate are determined for optimum growth and maintenance of a cell line of interest and the cell line of interest is then cultured in a continuous culture system using those set points. In addition, separate glucose delivery and pH and lactate maintenance set points are determined and used for maximum cell production if the cell line of interest is used to produce a biological product.

The method includes culturing a cell line of interest in a static culture and obtaining data for pH, glucose and lactate levels, cell count and biological product concentration, all with respect to time. Values for pH, glucose and lactate are determined at the maximum growth rate of the cell line of interest, the greatest increase in cell count, the maximum cell count and at a point of maximum production per cell of the biological product. These values are then used as set points to control pH, lactate and glucose in a continuous culturing system, such as a hollow fiber cartridge system, maximizing growth and providing conditions for optimum maintenance of the cells and, if desired, production of a biological product by the cells.

To improve growth of the cell line of interest in the continuous culturing system, pH level is maintained in the continuous culturing system corresponding to a pH value occurring at a point in time of greatest percentage increase in cell number in the static culture of the cell line of interest. Glucose and lactate in the continuous culturing system are maintained at concentrations corresponding to glucose and lactate concentrations at a point in time of greatest quantitative increase in cell number in the static culture of the cell line of interest.

If the cell line of interest is used to produce a biological product, the pH set point is changed when the cell count is at a maximum to a pH level corresponding to a pH value occurring at the maximum cell count in the static culture of the cell line of interest. In addition, the glucose and

lactate setpoints are changed in the continuous culturing system to concentrations corresponding to glucose and lactate concentrations at a time where greatest production per cell occurs in the static culture of the cell line of interest.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 and 2 are graphic views of kinetic data from an AFP-27 murine hybridoma cell line.

Figure 3 is a graphic view of the metabolic growth parameters with respect to time using the growth phase and the production phase set points of the present invention on the AFP-27 murine hybridoma cell line.

Figure 4 is a graphic view comparing metabolic growth parameters of the AFP-27 murine hybridoma cell line between the process of the present invention and a prior art process.

Figure 5 is a graphic representation of IgG production per day comparing the process of the present invention with the prior art process in an ACUSYST-P.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention includes a method of culturing cells in a continuous culturing system such as a perifusion system used in culturing cells in hollow fiber cartridges. The method of the present invention provides set point values for glucose, lactate and pH that maximize cell growth and set points for maintenance of and production by the cells if a useful biological product is to be produced by the cells.

The method of the present invention includes determining continuous culturing set points for pH, glucose and lactate from data generated by static culturing of the cell line of interest. Values for pH, glucose delivery and lactate production are then determined at the time of maximum growth rate of the cells, greatest increase in cell count, maximum cell count and greatest production of biological products on a per cell basis.

By "maximum growth rate" is meant the greatest percentage increase in cell number at a point in time. The growth rate is determined by the following equation:

$$\text{Growth rate} = LN(n2 / n1) / (s2 - s1)$$

where:

s2 = sample time t

s1 = sample time t-1

n2 = cell count at time t

n1 = cell count at time t-1

By the term "greatest increase in cell count" is meant the greatest numerical increase in cell count at a particular point in time. The greatest increase in cell count is found where the slope of a curve defined by a cell count curve generated by the static culture data of the cell line of interest is the highest. The slope is calculated by inserting the time value into the first derivative of the curve representing cell count.

By the term "greatest production of biological product per cell" is meant the quantity of product produced by the cell line of interest over a

given time period. Production per cell is calculated by the following equation:

Production per cell = (p2 - p1) / ((c2 - c1) / 2)

where:

p2 = product concentration at time t

p1 = product concentration at time t-1

c2 = cell count at time t

c1 = cell count at time t-1

When the point in time where the maximum growth rate, greatest increase in cell count and greatest production per cell per time are determined, the pH, glucose and lactose values for those points in time are determined from the static culture data.

For purposes of the present application, the continuous culture process is divided into two parts, the growth phase and the production phase.

By the term "growth phase" is meant that phase of culturing wherein the cells are increasing in number, the growth phase ending at a point wherein a curve defining the increase in cell number over time reaches a minimum slope.

By the term "production phase" is meant that phase occurring immediately after the growth phase wherein the cells are placed in an environment using glucose, lactate and pH levels for the specific purpose of maintenance of the cell population and for the production of a biological product and not for cell growth or proliferation.

For the growth phase of the process of the present application, the pH value occurring at the

time period wherein maximum growth rate occurs in the static culture is used as a pH set point in the continuous culturing system. The pH is maintained as close as possible to that pH value for ensuring maximum rate of growth of the cell line of interest.

Glucose delivery and lactate production are maintained at levels which are based on values either occurring at the time of maximum growth rate or at the time of greatest increase in cell count. If the cell count in the continuous system at the maximum growth rate of the static culture is too small for proper consumption of the glucose level, then the glucose value and lactate value issued at the point in time where greatest increase in cell number occurs in the static culture. Although the glucose level at the greatest increase in cell count is typically the choice, this is a matter of discretion depending on the particular cell line of interest.

In the production phase, the pH, glucose and lactate set points are changed at a point in time corresponding to maximum cell number in the continuous cell culturing system (i.e., cell count is at a maximum). A pH set point is chosen based on the pH value occurring at the point in time where the slope of the cell count curve is at a minimum in the static culturing system. Set point values for glucose and lactate are chosen based on their values when the production per cell in the static culture is at a maximum. These values occur in the static culturing system typically at a point in time after the cell count has reached a maximum.

The following example is illustrative only and is not intended to limit the present invention. The example is submitted in order to demonstrate more explicitly the process of the present invention.

EXAMPLE

Kinetic data from a static culture of AFP-27 murine hybridomas from a five-day static culture was used to generate set points for the production of monoclonal antibodies in an ACUSYST-P (Endotronics, Inc., Coon Rapids, Minnesota). Between 0.5 to 1.0 x 10^5 cells/ml were inoculated in T25 flasks using McCoy's 5A media. Aliquots were removed daily and assayed for glucose (VP Super System, Abbott Diagnostic Division), lactate (VP Super System, Abbott Diagnostic Division), cell number (Tripan Blue Stain), pH and product concentration (ELISA). The data resulting from the assays was used by a computer program to generate curves using the cubic spline curve-fitting method. The curves were used to determine the maximum growth rate, greatest increase in cell count, maximum cell count and greatest production rates per cell number for each hour of the static culture.

A typical hybridoma cell "fingerprint" from this computer model is illustrated in Figure 1. Referring to the cell count curve, cells grown in a static culture follow a predictable pattern of an initial lag phase and an exponential growth phase, slowing to a preliminary stationary phase, a stationary phase and a subsequent death phase. Glucose consumption as illustrated by the glucose curve follows an initial lag phase, followed by an

exponential phase concomitant with the cell growth phase. Lactate is produced in approximately a 1:1 ratio to glucose consumption. Lactate level is controlled by increasing or decreasing medium flow. As illustrated in Figure 2, pH is initially in the range of 7.35 to 7.45 and steadily decreases with time. Late in the death phase, pH shows a slight increase. As also illustrated in the curve in Figure 1, product accumulates proportionately to cell growth, but continues to increase even during the death phase. The increase in product concentration during the death phase is associated with increased production per cell, not with intracellular product released by lysed cells.

The factors to which the shift from stationary phase to death phase is commonly attributed are low nutrient levels, high metabolic waste products, low pH levels or cellular-produced feedback inhibition. The exact mechanism causing cell population to shift into the death phase is probably an intricate combination of all these factors and perhaps other factors, as yet undefined.

The essential amino acid concentrations were also tested to determine if their uptake rates follow the trend of the glucose uptake rate. The amino acid uptake rates appear to follow the glucose uptake rate and, surprisingly, tyrosine, methionine, valine and lysine were actually utilized very quickly along with L-glutamine for the AFP-27 murine hybridomas tested.

Table 1

Amino Acid Data for AFP-27 Cells Grown in McCoy's 5A Media Cell/ml is (x 10^5), amino acid data is in micromolar

Days	Cell/ml	ARG	LYS	GLN	THR	ALA	VAL	ILE	MET	HIS	GLU	TYR
0	1.17	91	43	905	28	76	37	56	17	56	69	18
1	2.33	86	30	786	11	95	32	47	14	33	66	14
2	9.11	76	19	438	11	101	16	25	6	34	67	5
3	22.20	48	7	331	6	163	9	22	4	31	51	0
4	3.78	46	6	262	6	214	10	20	3	29	29	0

Initially, set points for pH, glucose and lactate for culturing in the ACUSYST-P were selected at the apex of the growth curve as illustrated in Figures 1 and 2. Maintenance of such set points in the ACUSYST-P resulted in very unpredictable production of monoclonal antibodies from the hybridoma cells. Table 2 shows production data from four different hybridoma cell lines in the ACUSYST-P using hollow fiber cartridges.

Table 2

Production Data from an ACUSYST-P

Hybridoma Production Data

Cell Type	Product	Harvest Concentration (ug/ml)	Total (g/day)
mouse x mouse	IgA	5800	1.67
human x mouse	IgM	420	0.40
mouse x mouse	IgG	1000	0.50
human x mouse	IgG2a	1109	2.10

Total production ranged from 0.40 to 2.10 grams/day. In addition, when the production data was compared with static culturing in a T-flask, no correlation could be made. Another problem was that the production data for each hybridoma cell line was

not always repeatable from one production run to another. Using the same process control set point, production from the same cell line varied up to 400%.

Applicants have found that the pH value at the maximum growth rate of the cell line of interest in the static culture and the glucose and lactate levels at the greatest increase in cell count were found to provide a dramatic increase in cell growth in a hollow fiber cartridge perifusion system.

Applicants further found that for cell lines producing biological products, glucose, lactate and pH levels had to be changed since the environmental conditions that promote rapid cell growth do not maximize production of biological products by the cells. Effects of high levels of lactate on cells in static culture were observed and it was found that lactate inhibited the growth of cells, but did not affect viability. Also, high levels of lactate caused an increase in the production per cell. These results implied that a confluent culture could be inhibited from growing by high lactate levels allowing the culture to last a longer time. Additionally, the culture would potentially produce more biological products per cell. Therefore, the lactate and glucose production set points for use in the hollow fiber cartridge were selected at the point of highest production per cell based on static culture data. The pH value for the continuous culture set point was selected at the apex (minimum slope of the curve) of the cell count curve.

As illustrated in Figure 3, the metabolic growth parameters, glucose uptake rate and lactate uptake rate (G.U.R. and L.P.R.) showed that if the lactate, glucose and pH levels were controlled as indicated previously, the metabolic rates increased exponentially. Since metabolic rates are primarily dependent on a viable cell number, it is believed that the cell population in the hollow fiber cartridge increased at a similar rate. Thus, the doubling time of the cells in the hollow fiber cartridge system was about 22 hours, compared to 18 hours in the static culture system.

When the hollow fiber cartridge system was controlled at the production phase conditions, an opposite response was observed compared to the growth phase conditions. When the glucose, lactate and pH levels in the media were controlled at the production phase parameters, the cells repeatedly did not show any substantial increase in their metabolic rates, as indicated in Figure 3, implying a near constant viable cell population of 2×10^9 cells, based on metabolic rates. It is believed that the metabolic rates do not increase using the production phase set points since the death rate is approximately equal to the growth rate within the cell population. It is believed that this is true, since the viable cell density should have increased as the concentration of dead cells increased. This proposition is substantiated since there was no substantial increase in the visible cell mass.

Using this discovery, it is possible now to grow a cell line of interest to a high density quickly and then to shift to production set points that would keep the cells at or near a stable population. The approach of the present invention, in contrast to conventional production systems, would now allow for biological products to be removed sooner and at higher concentrations. A comparison of the metabolic rates using the process of the present invention compared to the rates of the prior art control conditions, described previously, using the ACUSYST-P and a hollow fiber cartridge for culturing cells are shown in Figure 4. As can be seen from Figure 4, during the growth phase, there is a substantial increase in the cellular rate of metabolism, and that after the cells had grown it was possible to maintain a highly viable culture, as compared to the prior art process.

Figure 5 illustrates the difference in production rates for the runs of Figure 4. In the culturing of the cells by the process of the present invention, there was an increase in production of about 100% over the cells that were cultured using the prior art set points.

Although the present invention has been described with reference to preferred embodiments, workers skilled in the art will recognize that changes may be made in form and detail without departing from the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A method of culturing a cell line of interest in a continuous culturing system, the method comprising:

maintaining a pH level in the continuous culturing system corresponding to a pH value occurring at a point in time of greatest percentage increase in cell number in a static culture of the cell line of interest; and

providing glucose in the continuous culturing system to the cell line at a concentration corresponding to glucose consumption at a point in time of greatest quanitative increase in cell number in the static culture of the cell line of interest.

1/5

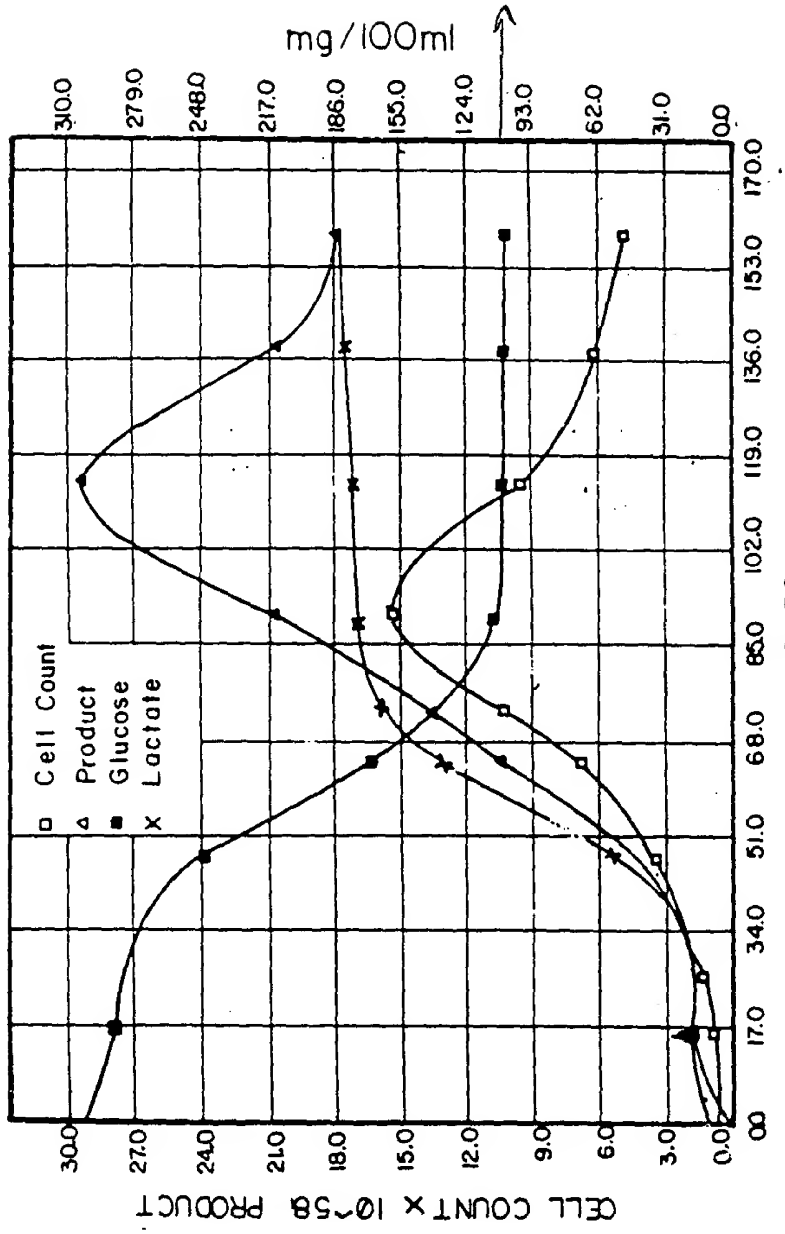
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Fig. 1

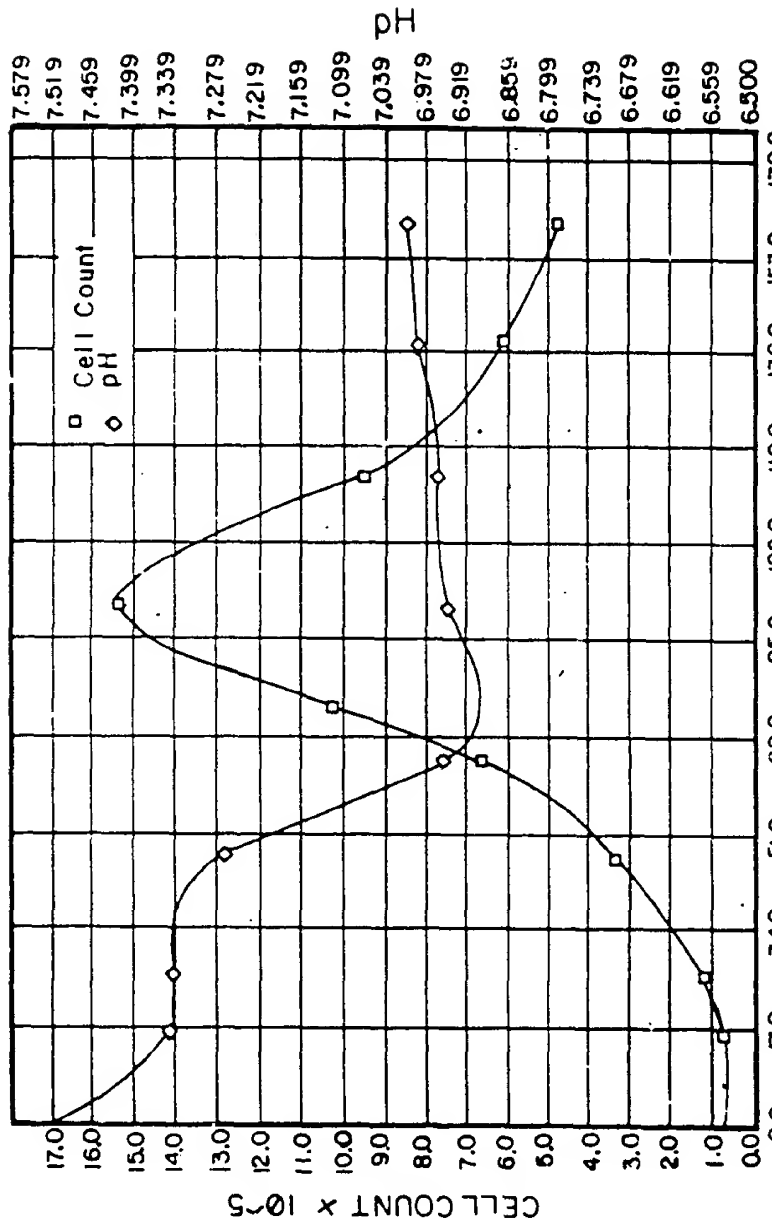
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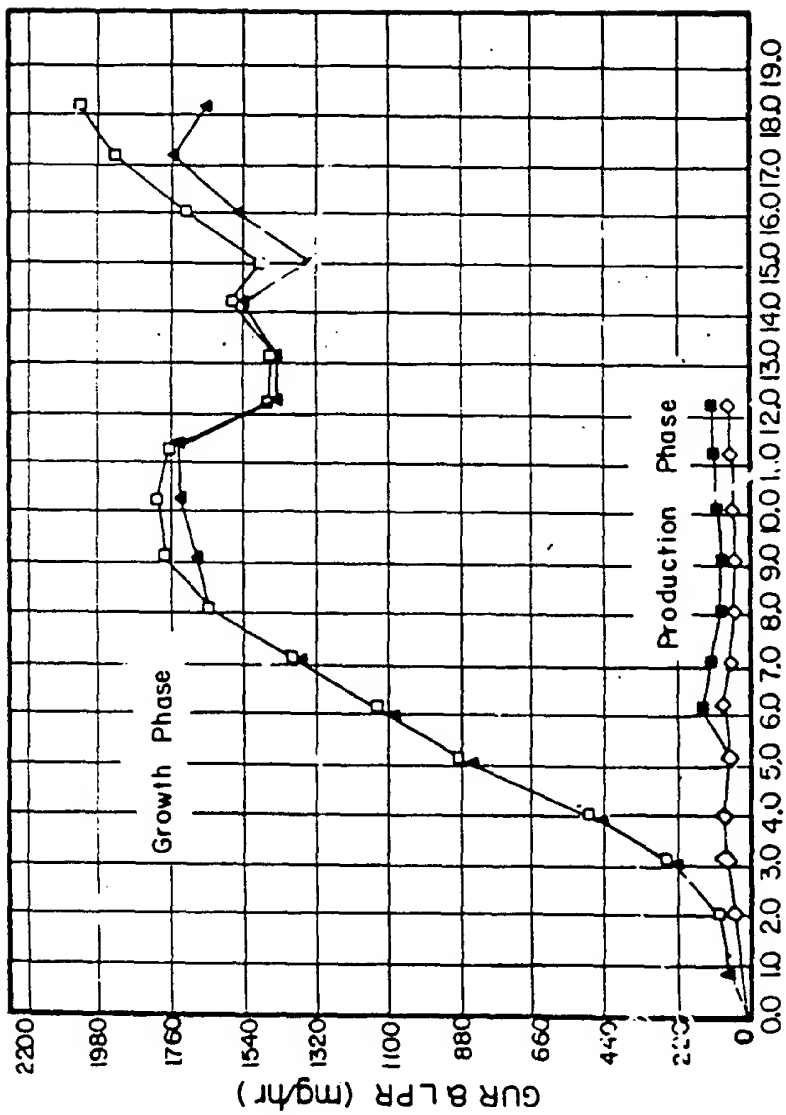
Fig. 2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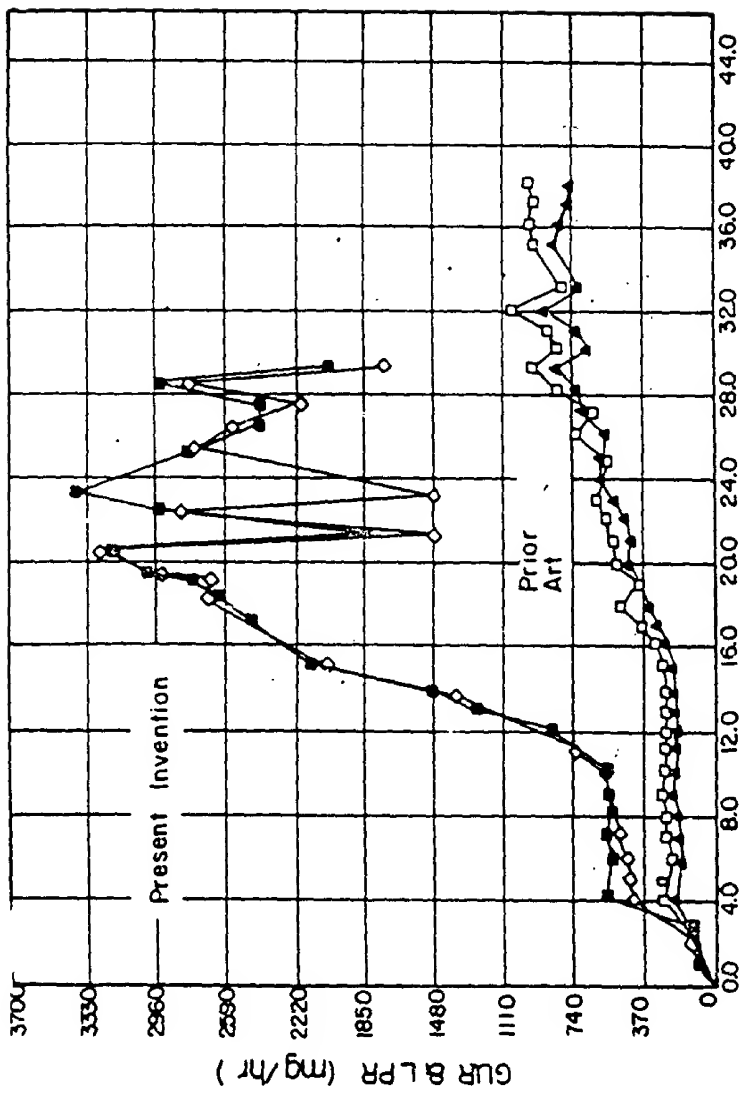
DAYS
Growth Phase
Production Phase

Fig. 3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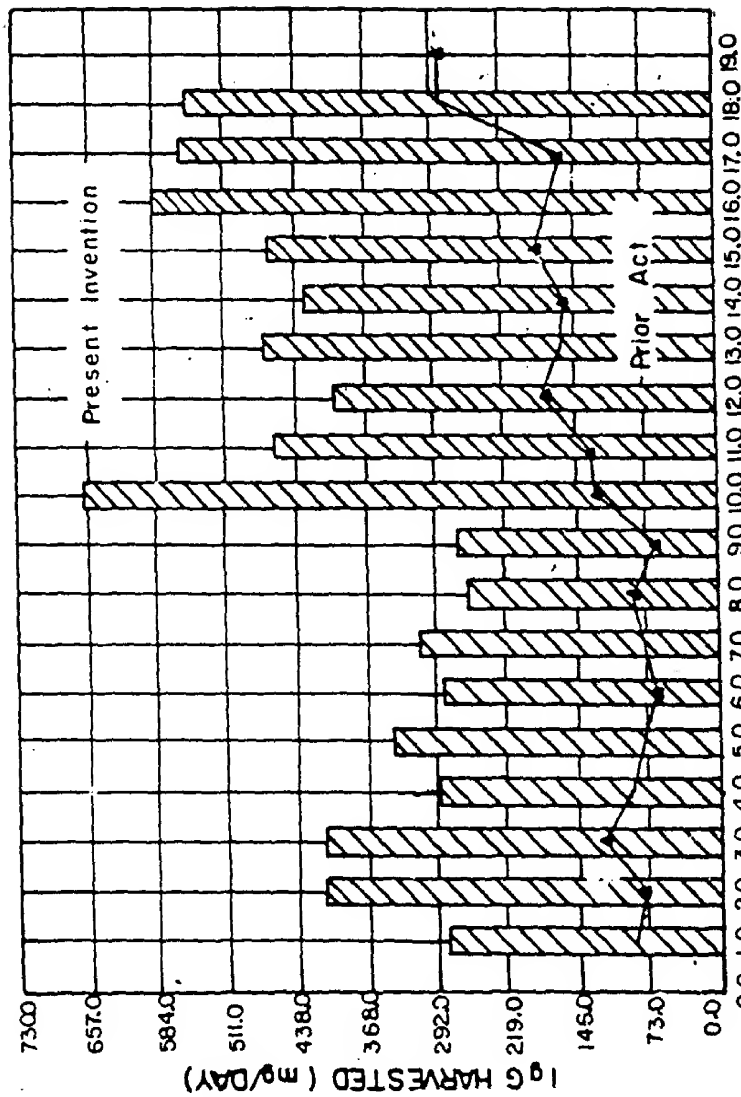
DAYS
Present Invention
Prior Art

Fig. 4


Present Invention
Prior Act
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1g G HARVESTED (mg/DAY)
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Fig. 5

INTERNATI NAL SEARCH REPORT

International Application No PCT/US87/02089

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12N 5/00
US CL : 435/240.1, 435/240.2, 435/240.21, 435/240.242

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System [5]	Classification Symbols
US	435/240.1, 240.2, 240.21,240.242

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [6]

DATA BASES: CHEMICAL ABSTRACTS SERVICE ON LINE (FILE CA, 1967-1987; FILE BIOSIS, 1969-1987)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [15]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,468,455 (HOPKINS) 28 August 1984, see the entire document.	1
Y	US, A, 4,444,882 (SHIMIZU) 24 April 1984, see the entire document.	1
Y	US, A, 3,941,662 (MUNDER) 2 March 1976, see the entire document.	1

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [1]	Date of Mailing of this International Search Report [1]
20 November 1987	09 DEC 1987
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	ELIZABETH HANLEY